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(54) Title: BIOLOGICALLY ACTIVE TGF- β 1 AND TGF- β 2 PEPTIDES			
(57) Abstract <p>The invention relates to peptides corresponding to regions of the amino acid sequence of TGF-β1 or TGF-β2 which retain, either in monomeric or polymeric forms, at least some of the biological activity of the respective full length TGF-β. The monomeric form of the peptide derived from TGF-β1 comprises the following amino acid sequence: CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP. The monomeric form of the peptide derived from TGF-β2 comprises the following amino acid sequence: CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA. Dimers may be formed via disulfide bonds between the amino-terminal cysteine residues, the carboxy-terminal cysteine residues, or amino- and carboxy-terminal cysteine residues of the monomer subunits.</p>			

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5 BIOLOGICALLY ACTIVE TGF- β 1 AND TGF- β 2 PEPTIDESTechnical field

 The present invention relates to biologically
active, peptides corresponding to regions of the amino
10 acid sequence of transforming growth factor β 1 or β 2 that
are capable of mimicking the activity of the respective
full length TGF- β s.

Background of the invention

15 PCT WO 84/001106, filed Sept. 23, 1983,
describes transforming growth factor β 1 (TGF- β 1) and its
use for the promotion of cell proliferation and tissue
repair, wound healing and treatment of trauma.

 U.S. Patent No. 4,843,063 describes two
20 cartilage inducing factors, CIF-A and CIF-B, found in
mammalian bone that (1) are cofactors for inducing
cartilage formation *in vivo*; (2) promote connective
tissue deposition *in vivo* in the absence of any added
activating agent or cofactor; and (3) are active in the
25 anchorage-independent cell growth assay used to
characterize TGF- β . The assay is referred to as the TGF-
 β assay herein and is described in Methods for
Preparation of Media, Supplements, and Substrate for
Serum-free Animal Cell Culture (1984) pp. 181-194, Alan
30 R. Liss, Inc.

 U.S. Patent No. 4,806,523, filed March 6, 1986,
discloses that CIF-A and CIF-B both possess anti-
inflammatory activity and are inhibitors of mitogen
stimulated T cell proliferation and B cell activation.
35 It also reports that CIF is localized in centers of

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hematopoiesis and lymphopoiesis and that CIF may, therefore, be useful for treating indications associated with malfunction or dysfunction of hematopoiesis or lymphopoiesis. CIF-A has since been shown to be
5 identical to TGF- β 1. CIF-B has since been recognized as a new form of β -type transforming growth factor and is now called TGF- β 2.

U.S. Patent No. 4,822,606, filed April 7, 1986, describes novel peptides having immunosuppressive or
10 immunoregulatory activity. These peptides are based on a 26 amino acid sequence which is highly conserved among retroviruses associated with immunosuppression.

European Patent Application 0 353 772 A2, filed August 4, 1988, discloses a method and compositions for
15 inhibiting proliferation of epidermal cells with TGF- β 1, TGF- β 2, or a fragment thereof.

U.S. Patent No. 5,061,786, filed May 25, 1989, discloses a biologically active peptide corresponding to residues 16-31 of TGF- β 1, with optional extensions of the
20 amino terminus.

Disclosure of the Invention

The invention relates to biologically active peptides corresponding to regions of the amino acid
25 sequence of TGF- β 1 or of TGF- β 2. The peptides retain and thus mimic the biological activities of mature, active TGF- β s. The peptides are thus suitable for use in any application for which TGF- β s are indicated. The peptides, either in monomeric or polymeric forms, retain
30 at least some of the biological activity of the respective full length TGF- β . The monomeric form of the peptide derived from TGF- β 1 comprises the following amino acid sequence: CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP. The monomeric form of the peptide derived from TGF- β 2
35 comprises the following amino acid sequence:

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CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA. Dimers may be formed via disulfide bonds between the amino-terminal, carboxy-terminal, or amino- and carboxy-terminal cysteine residues of the monomer subunits.

5 The present invention further provides compositions comprising the peptides of the invention and a pharmaceutically acceptable carrier therefor and methods of mimicking the effects of TGF- β s comprising administering to a patient an effective amount of such a
10 composition.

These and other embodiments of the present invention will be readily apparent to those of ordinary skill in view of the disclosure herein.

15 Brief Description of the Drawing

Figure 1 is a composite of line graphs depicting the results of the mink lung cell proliferation assay described in Example 2.

20 Detailed Description

Technical and scientific terms have been defined herein and this disclosure and the appended claims should be interpreted in view of those definitions. Unless specifically defined herein, all
25 other technical and scientific terms have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although other methods and materials similar or equivalent to those described may be used in the practice or testing of
30 the present invention, the preferred methods and materials are described herein.

It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural references unless the
35 context clearly dictates otherwise. Thus, for example,

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reference to "a peptide" or "an active peptide" includes mixtures of peptides of the general type described herein, and reference to "the method of administration" includes one or more methods of administration of the
5 general type described herein and/or of the type which will be readily apparent to those of ordinary skill in the art.

The methods for making the peptides employ, unless otherwise indicated, conventional techniques of
10 synthetic organic chemistry, protein chemistry, molecular biology, microbiology, and recombinant DNA technology, which are within the skill of one in the art. Such techniques are explained fully in the literature. See, e.g., Scopes, Protein Purification Principles and
15 Practices, 2d ed. (Springer-Verlag, 1987), Methods in Enzymology (Colowick and Kaplan, eds., Academic Press, Inc.); Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989, Handbook of Experimental Immunology,
20 Vols. I-IV, (Weir and Blackwell, eds) (Blackwell Scientific Publications, 1986); House, Modern Synthetic Reactions, 2nd ed., Benjamin/Cummings, Menlo Park, Cal., 1972. Atherton and Sheppard, Solid Phase Peptide Synthesis: A Practical Approach (Oxford University Press,
25 1989); Steward and Young Solid Phase Peptide Synthesis, 2nd ed. (Pierce Chemical Co., 1984).

All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference in their entirety.

30

A. Definitions

In defining the present invention, the following terms will be employed, and are intended to be defined as indicated below.

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As used herein, the amino acid sequence of TGF- β 1 is that described by Derynck et al., Nucl. Acids Res., 15:3188-3189 (1987). The amino acid sequence of TGF- β 2 is that described by Madisen et al., DNA, 7:1-8 (1988).

5 As used herein, the term "fibrinogenic" refers to factors that promote healing of damaged connective tissue resulting from injury inflicted by various types of trauma, inflammation, and immune reactions. These factors include but are not limited to those involved in
10 fibroblast chemotaxis, synthesis of collagen, hyaluronate, and tissue inhibitor of metalloproteinase (TIMP).

As used herein, the phrase "biologically active" refers to the ability to mediate a biological
15 function.

As used herein, the term "peptide" refers to an oligomer of at least two contiguous amino acid residues. Further, as used herein, the phrase "the peptides" refers to the peptides described herein unless otherwise
20 indicated.

As used herein, the term "treat" is intended to mean prophylaxis or attenuation of an existing condition. Accordingly, in the case of inflammation, the invention method may be used to prevent inflammation or alleviate
25 existing inflammation.

As used herein, the term "inflammation" is intended to encompass both acute responses (i.e. a response in which the inflammatory processes are active) and chronic responses (i.e. a response marked by slow
30 progress and formation of new connective tissue). Chronic and acute inflammation may be distinguished by the cell types involved. Acute inflammation often involves polymorphonuclear neutrophils; whereas chronic inflammation is normally characterized by a
35 lymphohistiocytic and/or granulomatous response.

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Examples of specific types of inflammation are diffuse inflammation, focal inflammation, croupous inflammation, interstitial inflammation, obliterative inflammation, reactive inflammation, specific inflammation, toxic inflammation, and traumatic inflammation.

As used herein, an amino acid sequence "substantially corresponding" to TGF- β 1 or TGF- β 2 will have at least 70% sequence homology with the amino acid sequence of TGF- β 1 or TGF- β 2, respectively, and retain functional activity. Further, it is preferable that any differences in sequence homology possessed by a peptide or protein having a "substantially corresponding" sequence are differences which do not adversely affect a desired property of that peptide or protein.

As used herein, the term "septic shock" refers to the sequence of events triggered by bacteremia during which cell wall substances (endotoxin in Gram-negative organisms and peptidoglycan/teichoic acid complex in Gram-positive organisms) activate the complement, kinin, and ACTH/endorphin systems. This series of metabolic events ultimately progresses to a state of shock.

As used herein, the term "patient" refers to animals including humans suffering from a condition that can be cured or ameliorated by a TGF- β .

25

B. General Methods

TGF- β s exhibit activity in the TGF- β assay described in Methods for Preparation of Media, Supplements, and Substrate for Serum-free Animal Cell Culture (1984) pp. 181-194, Alan R. Liss, Inc. The assay determines the ability to induce anchorage-independent growth in non-neoplastic normal rat kidney fibroblasts by measuring the formation of cell colonies in soft agar.

TGF- β s isolated to date are nonspecies specific as regards TGF- β activity. TGF- β s derived from other

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species are thus highly "homologous" in that they are similar in amino acid residue sequences and activity. These polypeptides are believed to have been highly conserved among animal species (i.e, wherein a given polypeptide from different mammalian species has an amino acid sequence that varies little, if at all, between species with respect to amino acid residue deletions, additives, or substitutions, and which variations do not adversely affect the non-species-specific activity of the molecule adversely) and have cross-species functionality. Accordingly, the peptides may be derived from cells or tissue of diverse animal origin or may be obtained by recombinant DNA technology. The peptides thus encompass the amino acid residue sequences of homologous TGF- β s. Correlatively, peptides from one vertebrate species may be used to treat another vertebrate species. The most common therapeutic uses of the peptides is in the treatment of patients such as humans, domestic animals such as cattle, sheep, and pigs, and sports or pet animals such as dogs, cats, and horses.

The peptides are useful in any application for which a TGF- β is indicated. The peptides mimic TGF- β activities including but not limited to inducing cartilage/bone formation and for repairing, replacing, or augmenting cartilage/bone tissue in animals including humans. Effective amounts of the peptides will normally be formulated with pharmacologically acceptable fluids or solid carriers. An effective amount of the peptides is an amount sufficient to evoke the biological response required to ameliorate the condition suffered by the patient.

The peptides may also be used in the same manner as other TGF- β s to promote (provoke and sustain) non-species specific cellular proliferation. Clinical applications of the cellular proliferation activity of

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these compositions include topical administration, for instance, for burn or wound healing or tissue repair. In such uses an effective amount of the peptides are formulated with pharmacologically acceptable carriers.

- 5 An effective amount of the peptides is an amount sufficient to induce soft tissue cell proliferation.

Topical dosage forms of the peptides can be formulated for instance as sprays, gels, ointments, or salves. Suitable dosage forms are those that are
10 pharmacologically acceptable and into which an amount of the peptides can be dispersed so as to allow diffusion of an effective amount of the peptides to the treatment site. The peptides, alone or in combination, may be dispersed into or incorporated into a polymeric substance
15 and coated onto implants. Such implants include, but are not limited to, collagenous soft and hard tissue implants, prostheses, sponges, wound dressings, and sutures. The peptides modulate local inflammatory responses to these foreign bodies and promote attachment
20 of the implants, particularly prostheses. Since such implants are often made from permeable materials peptides incorporated into the implants can diffuse from the implant to exert their properties.

The peptides may also be useful systemically,
25 for instance, for treating bone deficiencies, such as osteoporosis and osteopetrosis. For such treatment the peptides are formulated in therapeutically effective amounts with injectable carriers and administered parenterally to the patient. The dose is typically be in
30 the range of about 0.001 $\mu\text{g/kg}$ to 10 g/kg with a preferred range of about 100 $\mu\text{g/kg}$ to 1 g/kg .

Systemic dosage forms may be formulated for gastrointestinal administration (i.e., liquids, pills, tablets, or suppositories) or for parenteral injection.
35 The determination of dosages used in such applications

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are within the skill of one in the art and depend on factors such as the nature of the condition being treated, the size of the patient, and responsiveness to the peptides administered, and are therefore determined
5 by the caregiver.

The peptides may be used as oncostats in treating any type of cellular neoplasm, including, without limitation, carcinomas, myelomas, melanomas, and lymphomas. Particularly preferred targets are breast,
10 lung, colon, and ovarian carcinomas. The peptides may be administered locally or systemically, depending on the nature and degree of the neoplasm being treated. For local administration an oncostatically effective amount of the peptides or mixtures formulated thereof are
15 combined with a pharmacologically acceptable carrier and administered as a solid or semisolid implant which may or may not be of a sustained or a controlled release form. The peptides may also be formulated into an injectable for parenteral administration.

Alternatively, the peptides can be delivered to solid tumors in particular, including inoperable tumors, using current catheter technology for localized delivery via the arterial supply to the tumor. In this indication the peptides are mixed with a vasoocclusive agent, such
25 as injectable collagen, which would provide a means to reduce perfusion of the tumor and at the same time provide for the localized delivery of the peptides. Clips may also be used to occlude venous drainage, and thus maintain high doses of the peptides in the tumor
30 mass.

For systemic administration, oncostatically effective amounts of the peptides are formulated with pharmacologically acceptable carriers used for water soluble proteins including but not limited to
35 physiological saline, sugar solutions and the like for

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injection into circulation. Alternatively, the peptides may be formulated as a sustained release formulation to release the peptides into the circulation over a prolonged time period.

5 Specific targeting of the factor for tumor cells in systemic applications may be accomplished for instance by conjugation of a peptide to an antibody directed against tumor specific cell surface antigen(s). Enhanced tumor cell cytotoxicity may be accomplished by
10 covalently radiolabeling the fibrinogenic peptide with ^{131}I , a cytotoxic agent. The peptides are readily iodinated and retain full biological activity. Monoclonal antibody preparations with specificity for particular tumor types, such as breast and ovarian
15 tumors, are well known in the art. Other oncostats or chemotherapeutic drugs may be included in the formulation if desired.

 The term "oncostatically effective" is intended to indicate a dose that effects a significant (>50%)
20 inhibition of tumor cell proliferation. In *in vitro* assays, 50% inhibition is generally observed at TGF- β concentrations of the order of 0.2 ng/ml and saturation is achieved at 10 ng/ml. Inhibition may be monitored *in vivo* by monitoring the patient's tumor burden. The dose
25 of peptide that is oncostatically effective in a given treatment depend upon the patient, the type and degree of cancer being treated and the mode of administration. In general, the amounts administered to adult humans are in the range of about 0.001 $\mu\text{g/kg}$ to 10 g/kg. Corresponding
30 systemic administration involves the higher segment of the range (100 $\mu\text{g/kg}$ to 1 g/kg) due to clearance or other *in situ* inactivation of the polypeptide.

 The peptides are useful in the treatment of both local and systemic inflammation. When used as a
35 local anti-inflammatory agent, the peptides are usually

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formulated in effective amounts with pharmacologically acceptable carriers in weight ratios to carrier in the range of 1:1,000 to 1:20,000.

When used to treat inflammation at internal sites locally, the peptides, alone or in combination, may be injected, inhaled, placed surgically, or otherwise administered locally, depending on the particular formulation and the site where inflammation control is desired.

For systemic administration, the peptides may be formulated with conventional carriers used with water soluble proteins for injection into circulation. Alternatively, they may be formulated as a sustained release implant formulation if the indication being treated so requires.

Examples of formulations for systemic, topical, or parental administration may be found in Remington's Pharmaceutical Sciences, Gernnaro, ed., Mack Publishing Co., Easton, PA, 1985, hereby incorporated by reference in its entirety.

The amount of the peptides administered to treat inflammation depends upon the patient, the inflammatory condition being treated, and the mode of administration. In general, amounts administered to adult humans are in the range of about 1 mg to 10 g. When the peptides are administered locally, amounts in the lower portion of the range are normally used, typically 1 μ g to 100 mg. Correspondingly, systemic administration typically involves amounts in the 100 μ g/kg to 1 g/kg range.

The peptides may be particularly effective in the treatment of inflammation involving the respiratory system. In this application, the peptides may be administered by inhalation with a suitable aerosol. In this form, the peptides would be useful for the treatment

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of diffuse interstitial diseases of the lung including but not limited to, asbestosis, silicosis, or coal-miner's pneumoconiosis; the treatment of immunological diseases that involve the respiratory tract including, 5 but not limited to, rheumatoid arthritis, lupus erythematosus, or Goodpasture's syndrome; and the treatment of granulomatosis and eosinophilic granulomatosis.

The peptides may be combined with carriers in 10 the form of a salve, ointment, or other topical formulation and thereby be useful in the control of dermal inflammation by topical application. Such formulations would be particularly useful in the local treatment of skin conditions including, but not limited 15 to, psoriasis vulgaris, contact dermatitis, dermal ulcers, and acute or chronic eczematous dermatitis.

The peptides may be used either alone or combined with a slow release carrier and injected into or around joints, bone, or muscle for wound healing or 20 control of inflammation associated with various diseases. Such diseases include but are not limited to myositis (due to viral, bacterial, parasitic, fungal, or autoimmune processes); myasthenia gravis; osteomyelitis; osteoarthritis and rheumatoid arthritis.

Since TGF- β molecules have been shown to be 25 stable at low pH and resistant to enzyme digestion, these factors may be delivered gastrointestinally. The peptides are thus particularly useful for the treatment of gastrointestinal disorders including but not limited 30 to gastric and duodenal ulcers, granulomatous gastritis, esophagitis, enteritis and colitis.

TGF- β s have also been shown to be effective for the treatment of septic shock. International Publication Number WO 90/000903, filed July 21, 1989. The peptides 35 may be administered prophylactically or therapeutically,

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i.e., before, simultaneous with, or after an infection has set in. The peptides may be used to treat patients who are at-risk to bacterial infection, or who suffer from septicemia. Patients who are at risk include but
5 are not limited to those receiving immunosuppressive therapy and those suffering from severe thermal burns or other serious injury, cystic fibrosis, renal failure, or cancer, or who are undergoing extensive surgical procedures or organ transplantation.

10 The peptides may further be used to treat a patient for an indication associated with a dysfunction or malfunction of hematopoiesis or lymphopoiesis. The peptides may be administered to these patients by any suitable technique, including systemic or local, as
15 discussed above and will take into consideration the requirements of the individual patient, the method of administration, and other factors known to practitioners. Doses will typically be in the range of from about 100 μ g/kg to 1 g/kg.

20 The peptides may also be used to protect hematopoietic stem cells from the myelotoxicity of chemotherapeutic drugs, such as cyclophosphamide and mephalan, or radiation therapy. In such applications a therapeutically effective amount of the peptides will be
25 administered usually 3-72 hours prior to the administration of the chemotherapeutic drug or radiation therapy. The mode of administration is preferably interfemoral arterial, intraperitoneal, or subcutaneous, and is preferably by injection. Compositions and doses
30 may be formulated as discussed for the above applications, taking into consideration the requirements of the individual patient, the nature of the drug or radiation therapy used, the method of administration of the composition, and other factors known to

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practitioners. Doses will typically be in the range of 100 μ g/kg to 1 g/kg.

5 TGF- β may also be used in the prevention of severe cardiac injury resulting from reperfusion of ischemic myocardium. Lefer et al., Science, 249:61 (1990). The peptides thus may be administered, preferably intravenously or intracardiac, prior to or after the onset of ischemia. Peptide compositions and doses may be formulated as discussed for above applications, taking into consideration the requirements of the individual patient and other factors known to practitioners. Doses will typically be in the range of 100 μ g/kg to 1 g/kg.

15 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make the peptides, formulate the compositions and use them in connection with the invention and are not intended to limit the scope of the invention.

20

Example 1 - Peptide Synthesis

The following peptides were synthesized:

25 TGF- β ₁₆₋₃₁ corresponds to TGF- β 1 residues 16 through 31, and has the amino acid sequence
CVRQLYIDFRKDLGWK.

TGF- β ₂₁₆₋₃₁ corresponds to TGF- β 2 residues 16 through 31, and has the amino acid sequence
CLRPLYIDFKRDLGWK.

30 TGF- β ₁₆₋₄₇ corresponds to TGF- β 1 residues 16 through 47, and has the amino acid sequence
CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP.

TGF- β ₂₁₆₋₄₇ corresponds to TGF- β 2 residues 16 through 47, and has the amino acid sequence
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA.

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Peptides were synthesized on an Applied Biosystems model 431A peptide synthesizer using Fmoc amino acids (Applied Biosystems). Fmoc amino acids were coupled through reactions mediated with N,N-dicyclohexylcarbodiimide (DCC) or [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HBTU) in 1-hydroxybenzotriazole (HOBt). The peptide synthesis protocol was modified to double couple certain residues and to shorten the coupling times of certain residues to minimize premature termination of peptide synthesis by diketopiperazine formation. The peptides were synthesized on 4-hydroxymethylphenoxymethyl copolystyrene-1% divinylbenzene resin (HMP-resin). Reactive sites on the HMP-resin were capped with acetic anhydride following addition of the first residue.

The crude peptide (approximately 250 μ mol) was cleaved from the resin in 11.25 ml of 89% TFA, containing 0.75 g of crystalline phenol, 0.25 ml of ethylene dithiol, 0.5 ml of thioanisole, and water, at room temperature for over 1.5 hr. The resin was removed by filtration through a glass fritted disk and the filtrate was concentrated to 2 ml by using a rotoevaporator at 30°C. Crude peptide was precipitated by adding 50 ml of ice-cold diethyl ether and collected by filtration. The crude peptide was dissolved in 0.1% TFA, 30% acetonitrile and lyophilized. The dry peptide was stored at -20°C.

The dried crude peptide was dissolved in 0.1% TFA, 18% acetonitrile and chromatographed on a C18-reverse phase HPLC column (1 x 25 cm, Vydac, C18TP510). Peptides were eluted with a linear acetonitrile gradient in 0.1% TFA. The peak containing the desired peptide was identified by N-terminal sequencing and was confirmed by amino acid analysis. Protein concentration was determined with a BCA assay (Pierce, Rockford, IL). Reduced forms of cysteine residues were assayed using

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5,5'-dithio-bis-(2-nitrobenzoic acid). Ellman, Arch. Biochem. Biophys., 74:443 (1958).

To dimerize peptides, disulfide bonds were formed as follows. Purified peptide was dissolved at 5 mg/ml in 20% acetonitrile/80% dilute ammonium hydroxide (pH adjusted to 8.5 to 9.5 with 3% ammonium hydroxide). After stirring for 1 to 16 hr, precipitate that formed was dissolved by decreasing the pH to approximately 2 with 10% TFA. The reaction mixture was applied onto a C18-reverse phase HPLC column (1 x 25 cm, Vydac, C18TP510) and eluted with a linear acetonitrile gradient in 0.1% TFA. The dimeric forms of the peptides were identified by the shift in the elution position of the peak relative to the monomeric peptides. Monomeric and dimeric forms were confirmed by assaying for non-disulfide bonded cysteine residues and by mass spectroscopy.

Example 2 - Cell proliferation inhibition assay

TGF- β is known to inhibit proliferation of mink lung epithelial cells. The mink lung epithelial cell assay was thus used to test the ability of the peptides to mimic the biological activity of TGF- β .

Mink lung epithelial cells (ATCC, Rockville, MD, Mv1Lu CCL64) were plated at $0.5-1.0 \times 10^6$ cells/plate in 100 mm culture plates and grown in Eagle's minimal essential medium (MEM) supplemented with 50 units/ml penicillin, 50 μ g/ml streptomycin, nonessential amino acids, L-glutamine, and 10% fetal bovine serum (FBS). Cells were passaged while the cell density was subconfluent. Cells were detached with trypsin, collected by centrifugation at 800 x g for 2 min, and resuspended in the culture medium at 20,000 cells/ml. The cells were plated in 96-well microtiter plates at 1000 cells (50 μ l)/well.

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Peptides TGF- β ₁₁₆₋₃₁, TGF- β ₁₁₆₋₄₇, TGF- β ₂₁₆₋₃₁,
TGF- β ₂₁₆₋₄₇ in monomeric and dimeric forms were
lyophilized in the presence of sterile bovine serum
albumin (BSA) carrier. Samples (50 μ l) of the peptides
5 were dissolved in the cell culture medium and added to
the wells in triplicate. Recombinant human TGF- β ₂ was
used as the standard. The plates were incubated under a
5% CO₂/95% (V/V) air atmosphere at 37°C for 4 days.

The number of cells/well in this assay is
10 proportional to the activity of the constitutively
expressed enzyme, acid phosphatase. To measure acid
phosphatase activity, the wells were rinsed with
phosphate-buffered saline (PBS), filled with 100 μ l of
0.1 M sodium acetate, pH 5.5/0.1% Triton X-100/100 mM p-
15 nitrophenyl phosphate, and the plates were incubated at
37°C for 2 hr. Ten μ l of 1.0 N NaOH was added to each
well, and after 20 min at room temperature, the
absorbance at 405 nm was measured.

Some of the synthetic peptides and TGF- β ₂
20 inhibited proliferation of the mink lung epithelial cells
in a dose-dependant manner (Figure 1), as indicated by
the decrease in absorbance values. The inhibition curve
for TGF- β ₂ has been included in each figure for
comparison. Approximate concentrations at which a given
25 peptide and TGF- β ₂ inhibited cell proliferation half-
maximally (ED₅₀) are summarized in Table 1.

30

35

Table 1

	<u>Peptide</u>		<u>ED₅₀</u>
	TGF- β ₁ ₁₆₋₃₁	dimer	1-3 μ M
		monomer	not active
5	TGF- β ₂ ₁₆₋₃₁	dimer	not active
		monomer	not active
	TGF- β ₁ ₁₆₋₄₇	dimer	150 nM
		monomer	1 μ M
	TGF- β ₂ ₁₆₋₄₇	dimer	4-14 nM
		monomer	not active
10	TGF- β ₂		0.4-1 pM

TGF- β ₁₁₆₋₃₁ dimer inhibited proliferation of
 mink lung epithelial cells with an ED₅₀ of 1-3 μ M,
 comparable to levels of specific biological activity
 previously reported for this peptide. Chen et al., J.
 Bone Min. Res., 5: Abstract 26; and WO 90/14359. TGF-
 β ₂₁₆₋₃₁, a peptide homologous to the TGF- β ₁₁₆₋₃₁ peptide,
 was not active in either monomeric or dimeric form in the
 assay. The activity of the TGF- β ₁ synthetic peptide
 increased considerably when 16 additional carboxy
 terminal amino acid residues were added to form TGF- β ₁₁₆₋₄₇.
 The dimer of TGF- β ₁₁₆₋₄₇ exhibited an ED₅₀ of about
 150 nM. TGF- β ₁₁₆₋₄₇ peptide monomer exhibited some
 activity. In contrast to all the peptides mentioned,
 TGF- β ₂₁₆₋₄₇ peptide dimer exhibited an ED₅₀ of about 4-14
 nM. In comparison, bovine bone TGF- β ₂ inhibited
 proliferation of the cells with an ED₅₀ of 0.4 to 1.0 pM.
 BSA alone was inactive in this assay. Thus, dimerized
 TGF- β ₂₁₆₋₄₇ possesses about 11-38 fold more activity than
 dimerized TGF- β ₁₁₆₋₄₇. None of the monomeric peptides
 were active below 1 μ M.

It should be noted that activities of these
 peptides when assayed in the cell culture assay without
 prior lyophilization was comparable to the peptides co-
 lyophilized with BSA carrier prior to the assay.

Example 3 - Inhibition of murine thymocyte proliferation

In order to determine the effect of the peptides on proliferation of cell types other than mink lung epithelial cells, murine thymocytes were exposed to the peptides. Proliferation was measured by determining the amount of ^3H -thymidine incorporated into the thymocyte DNA in response to the peptides. The cell culture assay was performed as described by Ellingsworth et al., Cell. Immunol., 114:41-54 (1988). Single-cell suspensions of thymocytes were prepared from 4 to 8 week old C3H/HeJ mice and the cells were suspended in Eagle's MEM supplemented with 5% fetal calf serum (FCS), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine and 50 μM β -mercaptoethanol to 6.66×10^6 cells/ml. The thymocytes were plated into 96-well microtiter plates at 10^6 cells/well (150 μl). The cells were activated with 1.0 $\mu\text{g}/\text{ml}$ of phytohemagglutinin and 8 units/ml of interleukin-1 (IL-1) (Boehringer Mannheim, Indianapolis, IN). 0.02 to 200 pmol of the peptides or 0.02 to 50 fmol of TGF- β 2 were added and the total volume was adjusted to 250 μl with the culture medium. The cells were incubated at 37°C for 72 hr in a humidified incubator in 5% CO_2 -95% air atmosphere. The cells were pulsed with 0.5 $\mu\text{Ci}/\text{well}$ of ^3H -thymidine 24 hours prior to cell harvest. The thymocytes were harvested onto glass fiber filters and dried. The amount of ^3H -thymidine incorporation was determined by liquid scintillation counting method.

The results obtained indicated that TGF- β 2 inhibited incorporation of ^3H -thymidine into the DNA of murine thymocytes with an ED_{50} of approximately 4 pM. The TGF- β 2₁₆₋₄₇ dimer was also active as an inhibitor of thymocyte proliferation in this assay with an ED_{50} of 57 nM. However, both TGF- β 1₁₆₋₃₁ dimer and TGF- β 2₁₆₋₃₁

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dimer did not affect ^3H -thymidine incorporation at concentrations up to $0.8\ \mu\text{M}$.

Example 4 - TGF- β receptor binding assay

5 Rat muscle myoblasts (L6, American Type Culture Collection) were plated in 6-well culture plates and cultured in DMEM supplemented with 100 units/ml penicillin, $100\ \mu\text{g/ml}$ streptomycin, $2\ \text{mM}$ L-glutamine, 10% FBS until the cells were 70-90% confluent. Medium was
10 aspirated from the wells; and 1 ml of ice cold $20\ \text{mM}$ glycine, $135\ \text{mM}$ NaCl, pH 3.0 was added to each well for 2 min. The buffer was aspirated and each well was rinsed 2 times with 1 ml of DMEM, $25\ \text{mM}$ Hepes, pH 7.4, 0.1% BSA. Samples of TGF- β 1 and TGF- β 2 (3 to $1600\ \text{pM}$) and of
15 peptides (0.1 to $30\ \mu\text{M}$), each containing $25\ \text{pM}$ ^{125}I -TGF- β 1 or ^{125}I -TGF- β 2 were prepared in DMEM, $25\ \text{mM}$ Hepes, pH 7.4, 0.1% BSA and added in $500\ \mu\text{l}$ aliquots to the wells. Radiolabeled TGF- β samples ($500\ \mu\text{l}$) either without
20 unlabeled TGF- β or with excess unlabeled TGF- β (20 - $25\ \text{nM}$) were prepared and added to other wells. After 1 hr incubation at room temperature, each well was rinsed 2 times with 1 ml of Hank's balanced saline solution, 0.1% BSA. To each well, $500\ \mu\text{l}$ of 1% Triton X-100, 10% glycerol, $20\ \text{mM}$ Hepes, pH 7.4, 0.01% BSA was added and
25 incubated at 37°C with rotation for 30 min to lyse the cells. Samples were transferred into gamma-counting vials and counted for 1 min each. The results are presented in Table 2.

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Table 2

Ability of the peptides to compete
against binding of ^{125}I -TGF- β to L6
rat muscle myoblasts

5	<u>Peptide</u>	<u>^{125}I-TGF-β</u>	<u>ED₅₀</u>
	TGF- β 1	TGF- β 1	10-30 pM
	TGF- β 2	TGF- β 2	100-130 pM
	TGF- β 1 ₁₆₋₃₁ dimer	TGF- β 1, TGF- β 2	10-15 μM
10	monomer	TGF- β 1, TGF- β 2	not active
	TGF- β 1 ₁₆₋₄₇ dimer	TGF- β 1	not active
	monomer	TGF- β 1	not active
	TGF- β 2 ₁₆₋₃₁ dimer	TGF- β 2	30 μM
	monomer	TGF- β 2	not active
15	TGF- β 2 ₁₆₋₄₇ dimer	TGF- β 2	3 μM
	monomer	TGF- β 2	30 μM

The results obtained indicate that TGF- β 1 competed against binding of ^{125}I -TGF- β 1, and TGF- β 2 competed against binding of ^{125}I -TGF- β 2 for L6 rat muscle myoblasts, as previously reported by Segarini et al., J. Biol. Chem., 262:14655-14662 (1987). From the competitive binding data, the apparent dissociation constants were estimated to be 10-30 pM for TGF- β 1 and 100-130 pM for TGF- β 2. TGF- β 1₁₆₋₃₁ and TGF- β 2₁₆₋₄₇ peptide monomers, and TGF- β 1₁₆₋₄₇ and TGF- β 2₁₆₋₃₁ peptide monomers and dimers at concentrations of up to 30 μM were found to be either inactive or weakly active in competing against the binding of ^{125}I -TGF- β 1 or ^{125}I -TGF- β 2. However, TGF- β 2₁₆₋₄₇ dimer competed against the binding of ^{125}I -TGF- β 2 with an apparent dissociation constant of approximately 3 μM . TGF- β 1₁₆₋₃₁ peptide dimer competed against the binding of ^{125}I -TGF- β 1 with an apparent dissociation constant of 10-15 μM .

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Example 5 - Localization of TGF- β ₂₁₆₋₄₇ peptide activity and the disulfide bonds

To localize the regions of the TGF- β ₂₁₆₋₄₇ peptide responsible for biological activity, TGF- β ₂₃₂₋₄₇ was synthesized and compared to TGF- β ₂₁₆₋₃₁. TGF- β ₂₁₆₋₃₁ peptide, the N-terminal half of the TGF- β ₂₁₆₋₄₇ peptide, was found to be inactive in the mink lung epithelial cell and thymocyte proliferation assays and in the receptor binding assay, performed as discussed above. The dimeric form of the TGF- β ₂₃₂₋₄₇ peptide was prepared by forming a disulfide bond at Cys₄₄ between the two monomeric peptides.

Table 3

15 Competition against ¹²⁵I-TGF- β ₂ in binding to L6 rat muscle myoblast TGF- β receptor

<u>Peptide</u>	<u>ED₅₀</u>
TGF- β ₂	60 pM
TGF- β ₂ ₃₂₋₄₇	not active
dimer monomer	not active

As shown in Table 3, the TGF- β ₂₃₂₋₄₇ peptide as monomer and as dimer at concentrations up to 30 μ M did not compete against the binding of ¹²⁵I-TGF- β ₂ to the L6 rat muscle myoblasts. Furthermore, as shown in Table 4, the peptide as monomer and dimer at concentrations up to 10 μ M did not inhibit the proliferation of mink lung epithelial cells. Since TGF- β ₂₁₆₋₃₁ and TGF- β ₂₃₂₋₄₇ peptides were not active in these assays, the activity of the TGF- β ₂₁₆₋₄₇ peptide resides in the full length peptide.

The TGF- β ₂₁₆₋₄₇ peptide contains two cysteine residues, Cys₁₆ and Cys₄₄. It has been demonstrated by assaying for the reduced form of cysteine, that the two monomers are linked by a single disulfide bond, rather

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than two disulfide bonds involving both Cys₁₆ and Cys₄₄. Dimerization could thus occur by a disulfide bond between Cys₁₆-Cys₁₆, Cys₄₄-Cys₄₄, or Cys₁₆-Cys₄₄. Two peptides, TGF- β ₂₁₆₋₄₇Ser₁₆ and TGF- β ₂₁₆₋₄₇Ser₄₄, in which either Cys₁₆ or Cys₄₄ had been substituted with serine respectively, were synthesized as described in Example 1. Each of these peptides was able to dimerize to like peptides only by forming Cys₁₆-Cys₁₆ or Cys₄₄-Cys₄₄ disulfide bonds. The results are shown in Table 4.

Table 4

Activities of TGF- β ₂₃₂₋₄₇ peptide and TGF- β ₂₁₆₋₄₇ peptides with Cys to Ser substitutions. Inhibition of proliferation of mink lung epithelial cells.

<u>Peptide</u>		<u>ED₅₀</u>
TGF- β ₂		1.6 pM
TGF- β ₂ ₃₂₋₄₇	dimer	not active
	monomer	not active
TGF- β ₂ ₁₆₋₄₇ Ser ₁₆	dimer	380 nM
	monomer	1.3 μ M
TGF- β ₂ ₁₆₋₄₇ Ser ₄₄	dimer	450 nM
	monomer	not active

The results obtained in Table 4 indicate that TGF- β ₂₁₆₋₄₇Ser₁₆ peptide monomer and dimer inhibited the proliferation of mink lung epithelial cells with an ED₅₀ of 1.3 μ M and 380 nM, respectively. While TGF- β ₂₁₆₋₄₇Ser₄₄ peptide dimer inhibited cell proliferation with an ED₅₀ of 450 nM, the peptide monomer was inactive at concentrations of up to 10 μ M.

Thus, these Ser-substituted peptides are significantly less active than TGF- β ₂₁₆₋₄₇ peptide dimer in inhibiting proliferation of mink lung epithelial cells. These results suggest that the peptide dimer with

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disulfide bond between Cys₁₆ of one peptide and Cys₄₄ of the other peptide is the form that is most active in inhibiting the proliferation of mink lung epithelial cells with ED₅₀ in the range of 4-14 nM.

5 Thus, peptides with the biological properties of TGF- β have been disclosed. Although the preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the
10 scope of the invention as defined by the appended claims.

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Claims

1. A peptide comprising the amino acid
sequence CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA and homologs
5 and polymeric forms thereof wherein the monomeric form of
the peptide is less than the full length TGF- β 2 sequence.

2. A composition comprising the peptide of
claim 1 and a pharmacologically acceptable carrier
10 therefor.

3. A peptide consisting essentially of the
amino acid sequence CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA.

4. A composition comprising the peptide of
claim 3 and a pharmacologically acceptable carrier
15 therefor.

5. A homodimeric peptide comprising a
20 monomeric amino acid sequence of
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA and homologs thereof
wherein the monomers are linked via at least one
interpeptide disulfide bond between at least one set of
cysteine residues.

25 6. A composition comprising the peptide of
claim 5 and a pharmacologically acceptable carrier
therefor.

30 7. The peptide according to claim 5
comprising the amino acid sequence
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA
|
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA

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wherein the amino terminal cysteine residues are linked via a disulfide bond.

8. A composition comprising the peptide of claim 7 and a pharmacologically acceptable carrier therefor.

9. The peptide according to claim 5 comprising the amino acid sequence

10 CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA
CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA

wherein the carboxy terminal cysteine residues are linked via a disulfide bond.

15 10. A composition comprising the peptide of claim 9 and a pharmacologically acceptable carrier therefor.

20 11. The peptide according to claim 5 comprising the amino acid sequence

CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA

CLRPLYIDFKRDLGWKWIHEPKGYNANFCAGA

25 wherein the amino terminal cysteine residue of one monomer is linked to the carboxy terminal cysteine residue of the other monomer.

30 12. A composition comprising the peptide of claim 11 and a pharmaceutically acceptable carrier therefor.

35 13. A method of mimicking the effects of TGF- β s comprising administering to a patient an effective amount of the composition of claim 2 or 6.

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14. A peptide comprising the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP homologs and polymeric forms thereof wherein the monomeric form of the peptide is less than the full length TGF- β 1 sequence and greater than about twenty amino acid residues.

15. A composition comprising the peptide of claim 14 and a pharmacologically acceptable carrier therefor.

16. A peptide consisting essentially of the amino acid sequence CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP.

17. A composition comprising the peptide of claim 16 and a pharmacologically acceptable carrier therefor.

18. A homodimeric peptide comprising a monomeric amino acid sequence of CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP and homologs thereof wherein the monomers are linked via at least one interpeptide disulfide bond between at least one set of cysteine residues.

19. A composition comprising the peptide of claim 18 and a pharmacologically acceptable carrier therefor.

20. The peptide according to claim 18 comprising the amino acid sequence
CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP
|
CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP
wherein the amino terminal cysteine residues are linked via a disulfide bond.

21. A composition comprising the peptide of claim 20 and a pharmacologically acceptable carrier therefor.

5 22. The peptide according to claim 18
comprising the amino acid sequence
CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP
CVRQLYIDFRKDLGWKWIHEPKGYHANFCLGP
10 wherein the carboxy terminal cysteine residues are linked
via a disulfide bond.

23. A composition comprising the peptide of claim 22 and a pharmacologically acceptable carrier therefor.

24. The peptide according to claim 18 comprising the amino acid sequence

CVRQLYIDFRKDLGWKWIHEPKGYPHANFCLGP

_____|

20 | CVRQLYIDFRKDLGWKWIHEPKGYPHANFCLGP

wherein the amino terminal cysteine residue of one monomer is linked to the carboxy terminal cysteine residue of the other monomer.

25 25. A composition comprising the peptide of
claim 24 and a pharmaceutically acceptable carrier
therefor.

26. A method of mimicking the effects of TGF- β s comprising administering to a patient an effective amount of the composition of claim 15 or 19.

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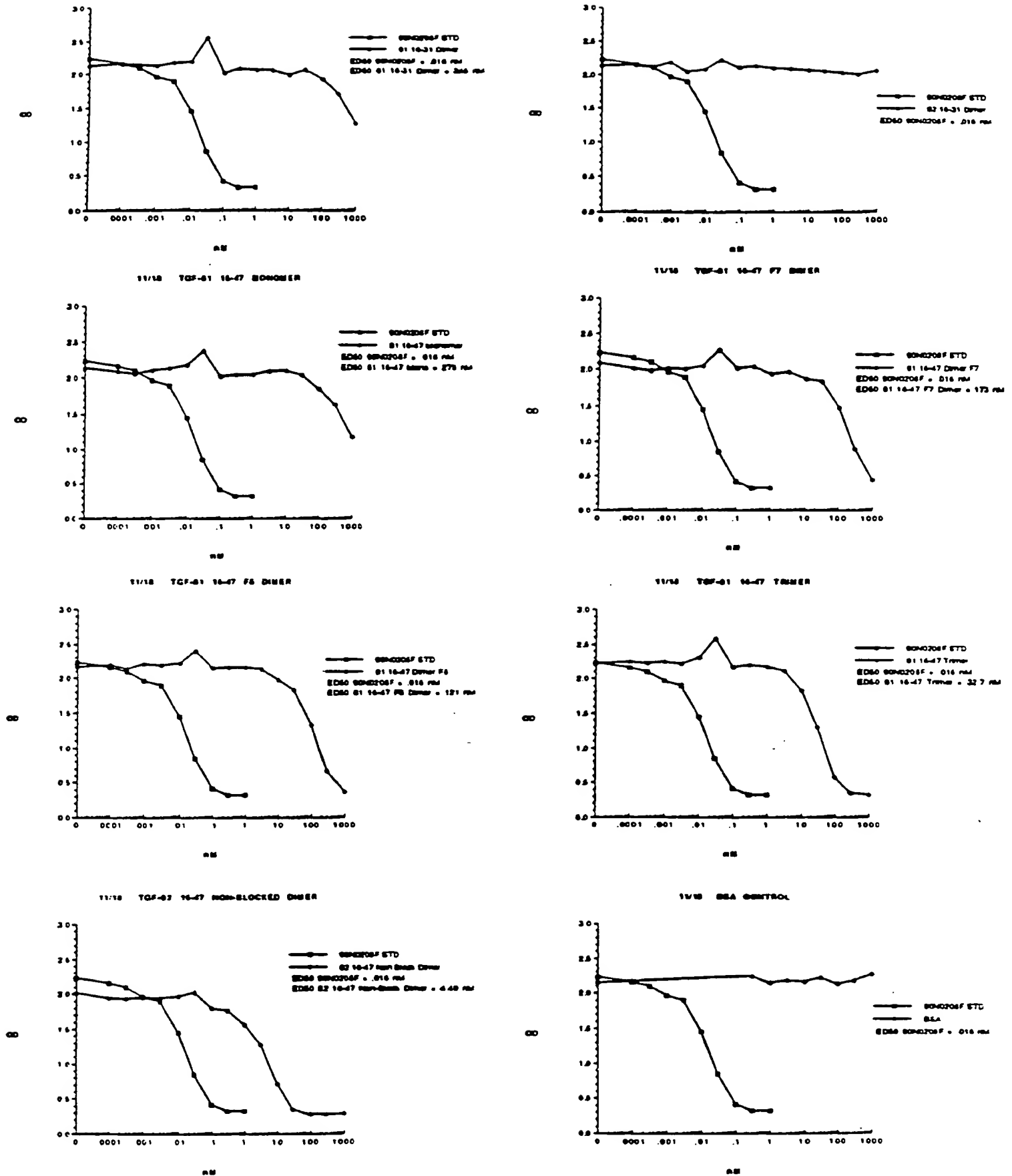


FIGURE 1

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